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Morphological and molecular characterization of *Phytophthora* species associated with root and crown rot of pomegranate in Iran

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Abstract

The purpose of this research was to identify the pathogens causing root and crown rot in major pomegranate-growing areas of Iran. Infected tissue samples were collected from trees with symptoms from 49 pomegranate orchards in four provinces of Iran: Fars, Markazi, Isfahan, and Kohgiluyeh va Boyer-Ahmad. In total, 23 *Phytophthora* spp. isolates were obtained, which were identified as *P. cryptogea* species complex (12 isolates) and *P. cinnamomi* (11 isolates) based on morphological characters. Molecular confirmation of identification was performed by inference of phylogeny of ITS-rDNA regions, β-tubulin gene, and the mitochondrial gene cytochrome c oxidase subunit 1. The results of our phylogenetic analysis confirmed the morphological identification of *P. cinnamomi* isolates and placed them in Clade 7c of *Phytophthora*. In addition, the *P. cryptogea* species complex isolates, despite morphological similarities, were in fact four distinct species including *P. cryptogea* sensu stricto (two isolates), *P. pseudocryptogea* (one isolate), *P.* sp. *kelmania* (one isolate), and *P. erythroseptica* (six isolates). This is the first report of pomegranate root and crown rot caused by *P. cinnamomi* and *P. cryptogea* species complex.

KEYWORDS

coxI, Iran, ITS-rDNA, multigene phylogeny, root pathogen, *tub2*

1 | INTRODUCTION

Pomegranate (*Punica granatum*), is one of the most ancient cultivated fruits native to Asia (Levin, 1994). This plant is an important fruit crop in Iran and is commercially cultivated mainly in Fars, Markazi, Razavi Khorasan, Isfahan, Yazd, Kerman, and Kohgiluyeh va Boyer-Ahmad. The planted area of *P. granatum* in Iran is approximately 80,000 ha with annual production of 750,000 t, which comprises 28% of global production (Bazargani-Gilani et al., 2014).

Root and crown rot and plant decline are major limitations affecting commercial pomegranate cultivation in many areas in Iran. The first and only report of pomegranate decline in Iran is by Banihashemi (1998), who identified *Phytophthora cactorum* and *P. citrophthora* as the agents of pomegranate root and crown rots in Fars province. Pomegranate root and crown rot, decline, and wilt have been reported from other countries caused by several fungal genera. *Ceratocystis fimbriata* is reported to cause pomegranate wilt in India (Somasekhara, 1999) and China (Huang et al., 2003) as well as *Verticillium dahliae* in Greece (Tziros & Tzavella-Klonari, 2008), & *Coniella granati* is reported causing pomegranate root and crown rot in Greece, Turkey, and Italy (Cacciola & Gullino, 2019; Çeliker et al., 2012; Thomidis, 2015; Thomidis & Exadaktylou, 2011). In addition, *Phytophthora* spp. have been reported causing losses in pomegranate orchards in various countries. *P. nicotianae* was identified as the causal agent of pomegranate fruit rot in India (Erwin & Ribeiro, 1996; Khosla & Bhardwaj, 2013), *P. palmivora* was reported from fruit rot of pomegranate in India (Erwin & Ribeiro, 1996) and Greece (Markakis et al., 2017) and from pomegranate root and crown rot in Turkey

(Turkolmez et al., 2016), and *P. niederhauserii* was isolated from roots of pomegranate in Spain (Abad et al., 2014).

The species in the genus *Phytophthora* are capable of causing serious diseases on a broad range of host plants in natural and agricultural ecosystems worldwide (Martin et al., 2014). The discrimination of *Phytophthora* spp. has long been a controversial issue. Traditionally the identification of species within *Phytophthora* was based on morphological and physiological criteria (Stamps et al., 1990; Waterhouse, 1963). However, morphological and physiological variability among *Phytophthora* species, the need for trained experts in morphological identifications, and availability of DNA sequence data has led to several DNA-based identification methods in recent years (Yang et al., 2017). Different parts of the genome are used in molecular phylogeny-based taxonomy of the genus *Phytophthora* such as ITS-rDNA regions, cytochrome oxidase I and II (*cox1* and *cox2*) genes, β-tubulin (*tub2*), part of the nuclear heat shock protein 90 (*HSP90*), and *NADH1* genes (Blair et al., 2008; Martin et al., 2014; Yang et al., 2017).

Despite long cultivation of pomegranates in Iran, no major disease has been reported until 2018 and 2019 when the first serious disease symptoms were recorded, and no comprehensive study on causal agents of root and crown rot of this crop has been investigated. During 2018 and 2019, pomegranate trees with symptoms of wilting, discolouration of infected tissues, gradual decline, chlorotic foliage, production of black exudates around trunks, and dieback of the branches were detected in some orchards in Fars, Markazi, Isfahan, and Kohgiluyeh va Boyer-Ahmad provinces of Iran. The objectives of this study were to determine the fungal pathogens causing root and crown rot in major pomegranate-growing areas of Iran based on morphological characteristics, and to carry out molecular confirmation using phylogenetic analyses of the DNA sequence data from ITS-rDNA regions, *tub2*, and *cox1*.

2 | MATERIALS AND METHODS

2.1 | Orchard surveys and sample collection

During 2018 and 2019, surveys were conducted across 49 pomegranate orchards in the main pomegranate cultivation areas of Iran in which decline and expanding cankers with abundant gummosis symptoms were recorded, comprising Fars, Markazi, Isfahan, and Kohgiluyeh va Boyer-Ahmad (Figure 1). Sample collections were carried out between early spring and late winter. Sampling at each orchard varied according to the number of trees with symptoms observed. Approximately 5%–15% of the trees in orchards were showing symptoms. The affected trees used for isolations were mostly 5–10 years old, with a few trees being 13–15 years old. The samples were taken from regions of stem base, root crowns, and roots showing symptoms. An average of 45 trees were sampled per province with 20 tissue pieces sampled per tree. Samples were taken to the laboratory in polyethylene bags in coolers, and kept in the refrigerator until diagnostic laboratory isolations were performed.

2.2 | Isolation and identification of *Phytophthora*

Tissues with symptoms were washed under running tap water for 2 hr to remove soil particles, blotted dry with sterile paper towels, and 2–5 mm pieces were cut from margins of infected tissues without extra treatments. The sections were placed onto a semiselective medium, CMA-PARPH (corn meal agar [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 & Martin, 1986). The plates were incubated at 25 °C and examined daily until the mycelial growth was observed.

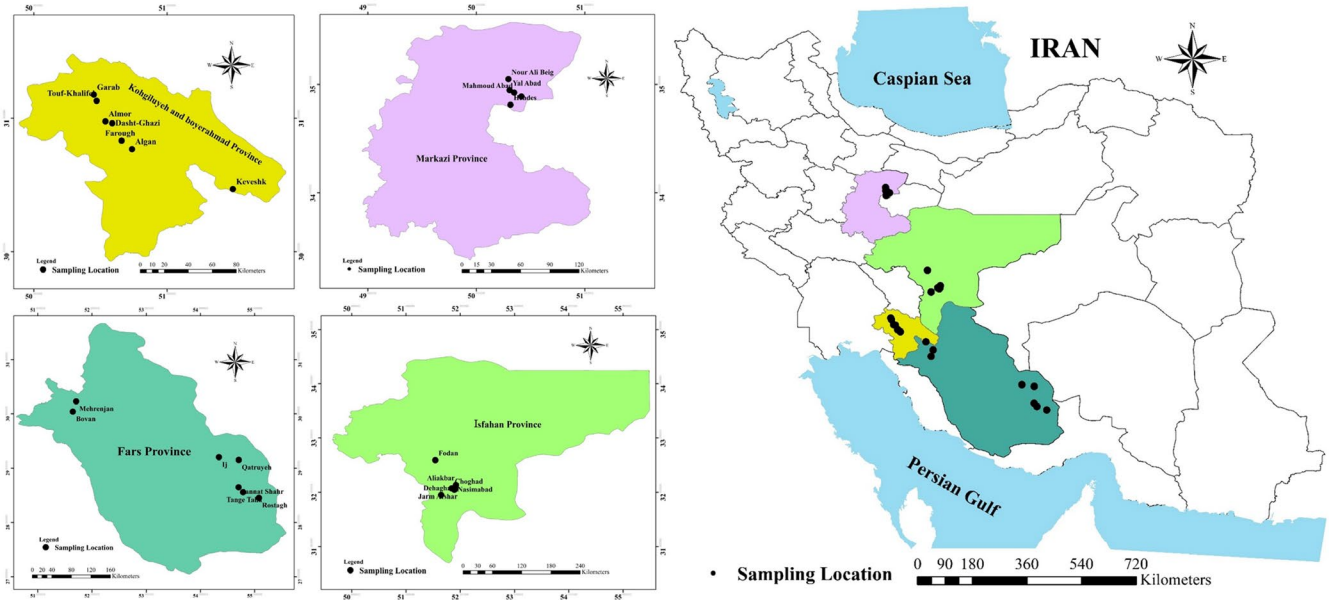

FIGURE 1 Map of Iran showing regions sampled for *Phytophthora* isolates, comprising Fars, Markazi, Isfahan, and Kohgiluyeh va Boyer-Ahmad provinces [Colour figure can be viewed at wileyonlinelibrary.com]

Purification of *Phytophthora* isolates was carried out using the single-zoospore method on water agar (WA) medium. In order to obtain zoospores, hemp seeds were placed on colonies of *Phytophthora* isolates grown on CMA-PARPH medium at 25 °C, and after 24 hr were transferred in 10 ml sterile distilled water to a plastic Petri plate for 2 days at 24 °C under white fluorescent light to produce sporangia. Then a 0.2 μl drop of the spore suspension from the released zoospores was placed on the surface of water agar (WA). Single germinating spores were transferred onto Petri dishes containing CMA.

The pure culture of isolates was used for morphological studies, including 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, 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 & Ribeiro, 1996; Stamps et al., 1990). Isolates were cultured on CMA slopes at 15 °C for long-term storage (Jeffers & Martin, 1986).

Isolates of heterothallic species were paired with themselves and with A1 and A2 mating type tester strains (Shiraz University culture collection, Iran). Cultures were incubated at 20 °C for 30–40 days in the dark. The presence of sexual structures was scored by direct examination under a light microscope. Fifty oogonia, oospores, and antheridia were measured using light microscopy.

2.3 | Pathogenicity on detached branches

Five isolates, P5, P13, P14, P15, and P19 (one from each species), were randomly selected and tested for their ability to cause symptoms on detached pomegranate branches. The detached stems from healthy and actively growing pomegranate trees were collected in June from orchards in Kohgiluyeh va Boyer-Ahmad province from sites not known to be diseased. The branches were trimmed to remove leaves and lateral shoots, and were cut to segments 18–22 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 (20–30 mm) were made in the bark with a scalpel. A 6 mm CMA plug from actively growing hyphae of each isolate 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 (Hajebrahimi & Banihashemi, 2011). The lesion development was examined after 6 weeks of incubation.

Necrotic lesion areas were measured using a leaf area meter tool (Fanavaran Alborz Andisheh Company). All experiments were performed in five replicates based on a randomized complete block design. Statistical analysis of the data was performed using SPSS v. 25 software and comparison of the mean of necrotic lesion area was carried out using Duncan's multiple range test.

2.4 | DNA extraction, PCR, and sequencing

Isolates were grown in V8-PARP broth at 20 °C for 7 days on a rotary shaker, the mycelium was harvested, and freeze-dried. Total DNA was extracted using the CTAB method according to Murray and Thompson (1980). The quality of the genomic DNA was checked on 1% agarose gel, visualized by staining with ethidium bromide solution, and the DNA stored at −20 °C until further analysis. The primers used for amplification of ITS-rDNA regions, and partial sequences of *tub2* and *cox1*, are listed in Table 1. PCR amplifications were carried out in a total volume of 20 μl consisting of 0.05 μM of each primer (CinnaGen), 1× Dream Taq buffer (MBI Fermentas), 0.4 μM dNTPs (MBI Fermentas), and 0.5 U Dream *Taq* DNA polymerase (MBI Fermentas). PCRs were performed in a Biometra thermocycler (Tpersonal) under the following conditions: an initial denaturation at 96 °C for 2 min; followed by 35 cycles of denaturation at 96 °C for 30 s, annealing step at the specific temperature for each primer pair (Table 1) for 40 s, extension step at 72 °C for 1 min; and a final extension at 72 °C for 10 min (Kroon et al., 2004). Successful amplification and the quality of PCR products were evaluated in 1% agarose gel, stained with ethidium bromide, and viewed under UV light (GelDoc, Bio-Rad

TABLE 1 List of primers used in this study

Primer	Sequence (5′–3′)	Region	Annealing temperature (°C)	Reference
ITS1	TCCGTAGGTGAACCTGCGG	ITS-rDNA	57	White et al. (1990)
ITS4	TCCTCCGCTTATTGATATGC			
COXF4N	GTATTTCTTCTTTATTAGGTGC	Cytochrome c oxidase subunit 1	52	Kroon et al. (2004)
COXR4N	CGTGAACTAATGTTACATATAC			
TUBUF2	CGGTAACAACTGGGCCAAGG	β-tubulin	60	Kroon et al. (2004)
TUBUR1	CCTGGTACTGCTGGTACTCAG			

Laboratories). PCR products were purified using a PCR purification kit (Fermentas) and sequenced in both directions at Macrogen (South Korea). The resulting sequence data were deposited in GenBank and the accession numbers are given in Table 2.

2.5 | Phylogenetic analysis

tub2, *cox1*, and ITS-rDNA sequences generated in this study, combined with sequences of representative taxa of Clades 7 and 8 from GenBank (Table 2), were used in the phylogenetic analyses to determine the taxonomic status of the pomegranate isolates.

The phylogenetic analyses of Clade 7 and Clade 8 were performed separately. Sequences were edited manually and aligned by Geneious v. 7 (Biomatters). The most appropriate model of sequence evolution was evaluated for each data set with jModelTest v. 2.1.4 using the Bayesian information criterion (BIC) for the following phylogenetic analyses. Multilocus phylogenetic Bayesian inference (BI) analyses were performed using MrBayes v. 3.2.2 (Ronquist et al., 2012). *tub2*, *cox1*, and ITS sequences were divided to three partitions. Four Markov chains 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 1,000 replicates to test the support of the branches. The resulting trees were viewed and edited in FigTree v. 1.4.0. *P. cryptogea* (P12, Table 2) and *P. cinnamomi* (P1, Table 2) were used as outgroups to root phylogenetic trees for members of *Phytophthora* Clades 7 and 8, respectively.

3 | RESULTS

3.1 | Morphological identification

The main disease symptoms were usually decline, foliar yellowing, branch dieback, and eventually tree mortality. The root crown regions and the aboveground parts of the trees up to approximately 1 m in height showed stem cankers with brown to dark-brown discolourations. The cankers and lesions in the trunk base were accompanied by gum, especially when the bark was removed. Cankers were only observed in the basal regions. Foliage symptoms, including wilting, gradual decline, discolouration, and chlorosis, were largely due to basal infections. The young pomegranate trees showing severe symptoms in some cases died (Figure 2).

Phytophthora species were efficiently recovered between May and September. From late winter through to early spring, no *Phytophthora* isolate was obtained from tissues with symptoms and cankers.

In total, 23 isolates of *Phytophthora* spp. were obtained from diseased trees in pomegranate orchards (Table 3). The highest isolation rates belonged to orchards of Isfahan (25%) followed by Kohgiluyeh va Boyer-Ahmad (18%), Fars (5%), and Markazi (5%) provinces. The isolation rates are calculated from the total number of *Phytophthora* isolates obtained from each province (Table 3) per total number of sampled trees per province (average 45). Based on morphological characters, isolates were identified as *P. cryptogea* species complex and *P. cinnamomi*.

Twelve isolates belonged to *P. cryptogea* species complex on the basis of morphological characteristics. The isolates were found on pomegranates in Markazi, Fars, and Kohgiluyeh va Boyer-Ahmad provinces in 2018–2019.

P. cryptogea isolates showed colonies with rose-shaped to chrysanthemum pattern on PDA and CMA (Figure 3a,b). Sporangia formed abundantly on liquid medium; they were terminal, insistent, nonpapillate, ovoid to pyriform, 41.5 ± 5.7 × 26.4 ± 3.4 μm. Sporangiophores were simple and unbranched, seldom sympodial (Figure 4a–c).

Oospores were aplerotic to nearly plerotic, spherical, with a mean diameter of 23.9 ± 5.8 μm, oospore wall thickness was 2.0 ± 0.5 μm. Oogonia were globose, 28.8 ± 3.0 μm in diameter and antheridia were amphigynous and terminal (Figure 4d,e). No chlamydospores were seen for any of the isolates. Hyphae were hyaline, normally nonseptate, 3.8–3.9 μm wide, with irregular hyphal swellings, which were more frequent in water (Figure 4f,g). The maximum, optimum, and minimum temperatures for colony growth on CMA were 35, 25, and 3 °C, respectively.

The results of phylogenetic analysis using a three-gene phylogeny (*tub2*, *cox1*, and rDNA-ITS) of the *P. cryptogea* species complex revealed that only two out of 12 were *P. cryptogea* sensu stricto while the rest of the isolates were *P. pseudocryptogea* (one isolate), *P.* sp. *kelmania* (one isolate), and *P. erythroseptica* (eight isolates). All *P. erythroseptica* isolates were homothallic in culture, while isolates of the other three species, *P. cryptogea*, *P.* sp. *kelmania*, and *P. pseudocryptogea*, were heterothallic. Indeed, all characters and dimensions overlapped for these four species except mating type system.

P. cinnamomi isolates were obtained from pomegranates in Isfahan province in 2018. Colony growth pattern was slightly chrysanthemum-like on PDA and radial on CMA (Figure 3c,d). Sporangia in liquid media had no papilla and were not shed; they were ellipsoid to ovoid, with a slight apical thickening, 56 × 32 μm. Sporangiophores were thin, usually unbranched (Figure 5a,b). All isolates were potentially heterothallic and were not able to produce oospores in single culture. Oospores were round, thin-walled (thickness 2 μm), with a mean diameter of 36 μm. Oogonia were globose, with smooth and thin walls, 22–27 μm in diameter. Antheridia were amphigynous, 17 × 22 μm (Figure 5c,d). Chlamydospores were globose, thin-walled, 8–80 μm in diameter, often in grape-like terminal or intercalary clusters of 3–10, separated from the mycelium by a basal septum (Figure 5e,f). Coralloid hyphae with frequent nodules, hyphal swellings in clusters, usually spherical, with a mean diameter of 43 μm, were observed (Figure 5g,h). The maximum, optimum, and minimum temperatures for colony growth on CMA were 34, 25, and 5 °C, respectively.

Based on morphological criteria combined with molecular data, the following species were identified (Table 3): *P. cinnamomi* from

TABLE 2 *Phytophthora* isolates used in phylogenetic analyses in this study

Species	Strain	GenBank accession no.		
		tub2	ITS	*cox1*
Phytophthora asiatica	CBS 133347	KX251666	MH620142	MH620062
Phytophthora austrocedrae	CPHST BL 5	MH493907	MG783380	KF358233
Phytophthora brassicae	PD_00072	EU080790	AF380148	AY564198
Phytophthora cajani	33D9	KX251673	KF358221	KF358234
Phytophthora cambivora	ATCC 46719	KX251495	KU681015	KU681020
Phytophthora cichorii	CBS 115029	KX252008	MH620159	MH620083
Phytophthora cinnamomi	P1	MN566568[a]	MN539989[a]	MN566591[a]
P. cinnamomi	P2	MN566569[a]	MN539990[a]	MN566592[a]
P. cinnamomi	P3	MN566570[a]	MN539991[a]	MN566593[a]
P. cinnamomi	P4	MN566571[a]	MN539992[a]	MN566594[a]
P. cinnamomi	P5	MN566572[a]	MN539993[a]	MN566595[a]
P. cinnamomi	P6	MN566573[a]	MN539994[a]	MN566596[a]
P. cinnamomi	P7	MN566574[a]	MN539995[a]	MN566597[a]
P. cinnamomi	P8	MN566575[a]	MN539996[a]	MN566598[a]
P. cinnamomi	P9	MN566576[a]	MN539997[a]	MN566599[a]
P. cinnamomi	P10	MN566577[a]	MN539998[a]	MN566600[a]
P. cinnamomi	P11	MN566578[a]	MN539999[a]	MN566601[a]
P. cinnamomi	CAS 144.22	KC609408	KC478663	MH620070
Phytophthora cryptogea	P12	MN566579[a]	MN540000[a]	MN566602[a]
P. cryptogea	P13	MN566580[a]	MN540001[a]	MN566603[a]
P. cryptogea	PD_00031	EU080447	HQ261549	AY659606
Phytophthora dauci	CBS 127102	KX252015	MH620160	MH620084
Phytophthora drechsleri	PD_00086	EU079507	HQ261553	AY659604
Phytophthora erythroseptica	P16	MN566583[a]	MN540004[a]	MN566606[a]
P. erythroseptica	P17	MN566584[a]	MN540005[a]	MN566607[a]
P. erythroseptica	P18	MN566585[a]	MN540006[a]	MN566608[a]
P. erythroseptica	P19	MN566586[a]	MN540007[a]	MN566609[a]
P. erythroseptica	P20	MN566587[a]	MN540008[a]	MN566610[a]
P. erythroseptica	P21	MN566588[a]	MN540009[a]	MN566611[a]
P. erythroseptica	P22	MN566589[a]	MN540010[a]	MN566612[a]
P. erythroseptica	P23	MN566590[a]	MN540011[a]	MN566613[a]
P. erythroseptica	61J2	KX251896	MH620153	MH620077
Phytophthora europaea	CBS 109049	KX251523	MH620138	MH620055
Phytophthora foliorum	PD_00110	EU079680	HQ261561	EU124918
Phytophthora fragariae	CBS 209,46	KX251544	MH620139	MH620058
Phytophthora fragariaefolia	CBS 135747	KX251854	MH620149	MH620073
Phytophthora hibernalis	CPHST BL 41G	MH493948	MG865506	MH620088
Phytophthora kelmania	P15	MN566582[a]	MN540003[a]	MN566605[a]
P. kelmania	ATCC MYA-4162	KX251987	MH620157	MH620081
Phytophthora lactucae	61F4	KX252043	MH620161	MH620085
Phytophthora lateralis	ATCC MYA-3898	KX252134	MH620165	MH620089
Phytophthora medicaginis	PD_00015	EU079900	HQ643273	KF358236
Phytophthora melonis	32F6	KX251694	EU088256	KF317110
Phytophthora nagaii	CBS 133248	KX251861	MH620150	MH620074

(Continues)

TABLE 2 (Continued)

Species	Strain	GenBank accession no.		
		tub2	ITS	*cox1*
Phytophthora niederhauserii	01D5	KX251715	GU230789	GU477617
Phytophthora obscura	CPHST BL 84	MH493989	MG865554	MH620091
Phytophthora parvispora	CBS 411.96	KC609407	KC478672	KC609418
Phytophthora pisi	60A4	KX251736	KT183042	MH620066
Phytophthora pistaciae	33D6	KX251749	KT183043	MH620067
Phytophthora primulae	CBS 620,97	KX252064	KF358226	KF358238
Phytophthora pseudocryptogea	P14	MN566581[a]	MN540002[a]	MN566604[a]
P. pseudocryptogea	CPHST BL 139	MH493929	KP288376	KP288342
Phytophthora ramorum	PD_00058	EU080684	HQ261662	EU124929
Phytophthora rubi	30D7	KX251551	KU899155	KU899310
Phytophthora sansomeana	ATCC MYA-4455	KX251931	MH620155	MH620079
Phytophthora sinensis	PD_00119	EU079750	HQ261671	AY564202
Phytophthora sojae	CBS312.62	KX251763	MH620145	MH620068
Phytophthora syringae	ATCC 34002	KX252197	MH620168	MH620092
Phytophthora trifolii	ATCC MYA-3901	KX251952	HQ643368	MH620080
Phytophthora uliginosa	CBS 109055	EU079693	KF358232	KF358244
Phytophthora uniformis	CPHST BL	MH493905	KU899221	KU899376
Phytophthora vignae	ATCC 46735	KX251777	MH620146	MH620069

[a]Sequences generated in this study.

FIGURE 2 Symptoms of crown and root rot caused by *Phytophthora* species on pomegranate trees: the aboveground regions at the trunk base up to approximately 1 m height showed (a) stem lesions with brown to dark-brown discolourations; and (b) dark lesions and discolourations at the crown [Colour figure can be viewed at wileyonlinelibrary.com]

Isfahan province (relative frequency 47.8%), *P. cryptogea* from Markazi province (8.7%), *P. erythroseptica* from Kohgiluyeh va Boyer-Ahmad (34.8%), and two species, *P. pseudocryptogea* and *P.* sp. *kelmania*, from Fars province (4.3%.). *P. cinnamomi* was obtained from stem bases and root crowns, *P. cryptogea* and *P. erythroseptica* were recovered from roots and crowns, and *P. pseudocryptogea* and *P.* sp. *kelmania* were isolated only from root crowns of infected trees.

3.2 | Pathogenicity

The obtained *Phytophthora* species—*P. cinnamomi*, *P. erythroseptica*, *P. cryptogea*, *P. pseudocryptogea*, and *P.* sp. *kelmania*—were pathogenic on detached branches of pomegranate 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

TABLE 3 Isolates of *Phytophthora* spp. obtained in this study during 2018–2019

Species	Isolate	Source	Location of isolation	No. of sampled orchards
P. cinnamomi	P1, P2	Stem base	Isfahan	12
P. cinnamomi	P3–P11	Root crown		
P. cryptogea	P12	Root	Markazi	7
P. cryptogea	P13	Root crown		
P. pseudocryptogea	P14	Root crown	Fars	15
P. sp. *kelmania*	P15	Root crown		
P. erythroseptica	P16, P17	Root	Kohgiloueh va Boyer-Ahmad	15
P. erythroseptica	P18–P23	Root crown		

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 all *Phytophthora* species were responsible for pomegranate root and crown rots in Iran.

Results of analysis of variance (ANOVA) of the necrotic lesions area in Table 4 showed that there was a significant difference between *Phytophthora* species ($F = 318.653$, $\alpha = .001$). Duncan's post hoc test showed that the five *Phytophthora* species had different virulence levels based on the lesion area (Table 4). Furthermore, the most and least virulent species on detached branches of pomegranate were *P. cryptogea* and *P.* sp. *kelmania*, respectively (Figure 6).

3.3 | Phylogenetic analysis

PCR amplification and sequencing were successful for all isolates. The obtained sequences of *Phytophthora* isolates were submitted to GenBank under the following accession numbers: MN566568 to MN566590 for *tub2*, MN566591 to MN566613 for *cox1*, and MN539989 to MN540011 for ITS-rDNA regions (Table 2). The aligned data sets of Clade 7 for *tub2*, *cox1*, and ITS-rDNA and Clade 8 for *tub2*, *cox1*, and ITS-rDNA consisted of 1,142, 1,293, 985, 1,204, 1,317, and 956 characters, respectively. The aligned multigene data set of Clade 7 taxa contained 3,420 characters of which 284 were informative, and for Clade 8 taxa, 3,477 characters of which 402 were informative. The two phylogenetic analysis methods, BI and MP, generated trees with similar topologies amongst species. The most appropriate model chosen by jModelTest based on BIC was the general time reversible nucleotide substitution model with gamma-distributed rate variation and a proportion of invariable sites (GTR+I+G). The topology and branch lengths of the phylogenetic inferences are shown in Figures 7 and 8.

Figure 7 shows the phylogenetic position of isolates P1 to P11 using a Bayesian analysis of the combined data set of *tub2*, *cox1*, and ITS-rDNA sequences. Isolates P1 to P11 resided in Clade 7c of *Phytophthora* and grouped with *P. cinnamomi* in a well-supported clade (posterior probability = 100). The multigene phylogenies of the combined data set of *tub2*, *cox1*, and ITS-rDNA sequences of isolates P12 to P23 revealed four separate lineages within Clade 8a of *Phytophthora*, corresponding to three described species, *P. cryptogea*, *P. erythroseptica*, *P. pseudocryptogea*, and an undescribed *Phytophthora*, *P.* sp. *kelmania* (Figure 8). Isolates P12 to P23 were designated as *P. cryptogea* in preliminary morphological analyses because of the many morphological characteristics corresponding to the original description of *P. cryptogea* by Pethybridge and Lafferty (1919). However, sequence analyses showed that isolates P12 and P13 grouped with *P. cryptogea* sensu stricto (posterior probability = 100). Isolate 14 grouped with *P. pseudocryptogea*, and isolate P15 corresponded to the undescribed *P.* sp. *kelmania*. Isolates P16 to P23 formed a monophyletic group with *P. erythroseptica* in a well-supported clade (posterior probability = 100).

4 | DISCUSSION

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; Zentmyer, 1983). The main goal of this study was to identify *Phytophthora* species causing pomegranate root and crown rots in four major pomegranate-growing areas of Iran. In this survey, *P. cinnamomi*, *P. cryptogea*, *P. erythroseptica*, *P. pseudocryptogea*, and *P.* sp. *kelmania* are reported to cause disease in pomegranate orchards of Iran. To the best of our knowledge, this is the first report of these species causing pomegranate root and crown rot in the world.

Until now, species of *Phytophthora* reported to infect pomegranate root and crown worldwide were *P. palmivora* (Markakis et al., 2017; Turkolmez et al., 2016) from Clade 4, *P. nicotianae* (Khosla & Bhardwaj, 2013) from Clade 1, and *P. niederhauserii* (Abad et al., 2014) from Clade 7b of *Phytophthora*, as well as *P. cactorum* from Clade 1a and *P. citrophthora* from Clade 2a of *Phytophthora* from Iran (Banihashemi, 1998). In Iran, the only survey on causal agents of pomegranate root and crown rot was attempted by Banihashemi (1998) and was limited to Fars province. This study represents the first extensive attempt to characterize fungal pathogens of major pomegranate-growing areas of Iran, and with pathogen identification based on morphological, pathogenicity, and phylogenetic analyses. Morphological and phylogenetic

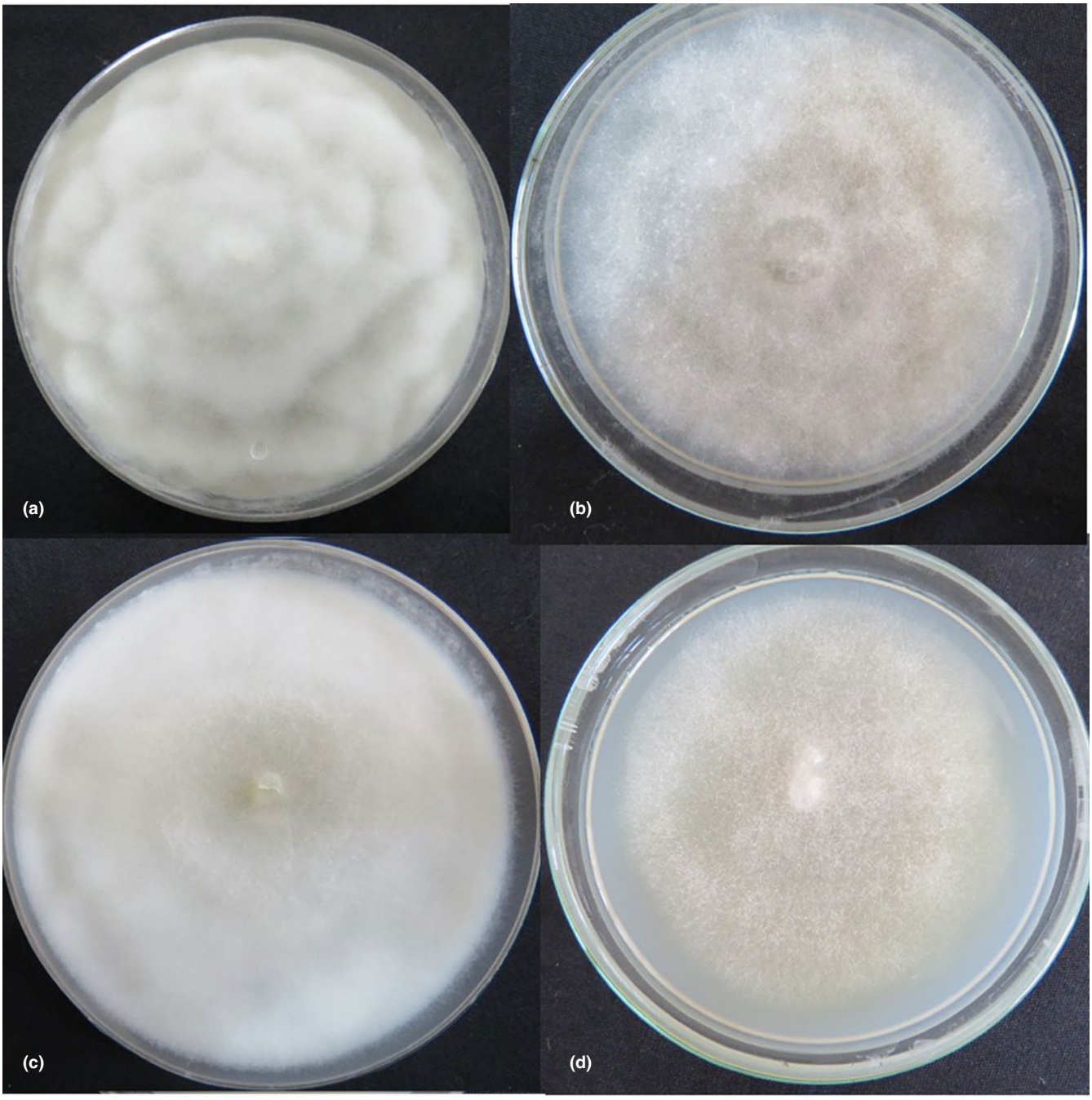

FIGURE 3 Colony morphology of two *Phytophthora* species after 10 days at 25 °C; fungal growth of *Phytophthora cryptogea* species complex on potato dextrose agar (PDA) (a) and cornmeal agar (CMA) (b). Fungal growth of *P. cinnamomi* on PDA (c) and CMA (d) [Colour figure can be viewed at wileyonlinelibrary.com]

analyses of the DNA sequence data of *tub2*, *cox1*, and ITS-rDNA allowed identification of one species from Clade 7c of *Phytophthora*, *P. cinnamomi*, and four species from Clade 8a of *Phytophthora*, *P. cryptogea*, *P. erythroseptica*, *P. pseudocryptogea*, and *P.* sp. *kelmania*, which are reported here as causal agents of root and crown rot of pomegranate in major pomegranate-growing areas of Iran.

The overall topologies of our phylogenetic trees of Clades 7 and 8 were consistent with previous studies (Martin et al., 2014; Yang et al., 2017). During morphological analyses, isolates P2 to P23 were identified as *P. cryptogea* species complex and isolates P1 to P11 were identified as *P. cinnamomi*. Our multigene phylogenetic analyses demonstrated that isolates P12 to P23, although morphologically similar according to the phylogenetic species concept, are in fact four distinct species closely related to *P. cryptogea*. In previous studies, *P. cryptogea*, *P. erythroseptica*, and *P. pseudocryptogea* have been reported to be morphologically similar (Safaiefarahani et al., 2015). The discrimination of these species has long been a controversial issue. Mostowfizadeh-Ghalamfarsa

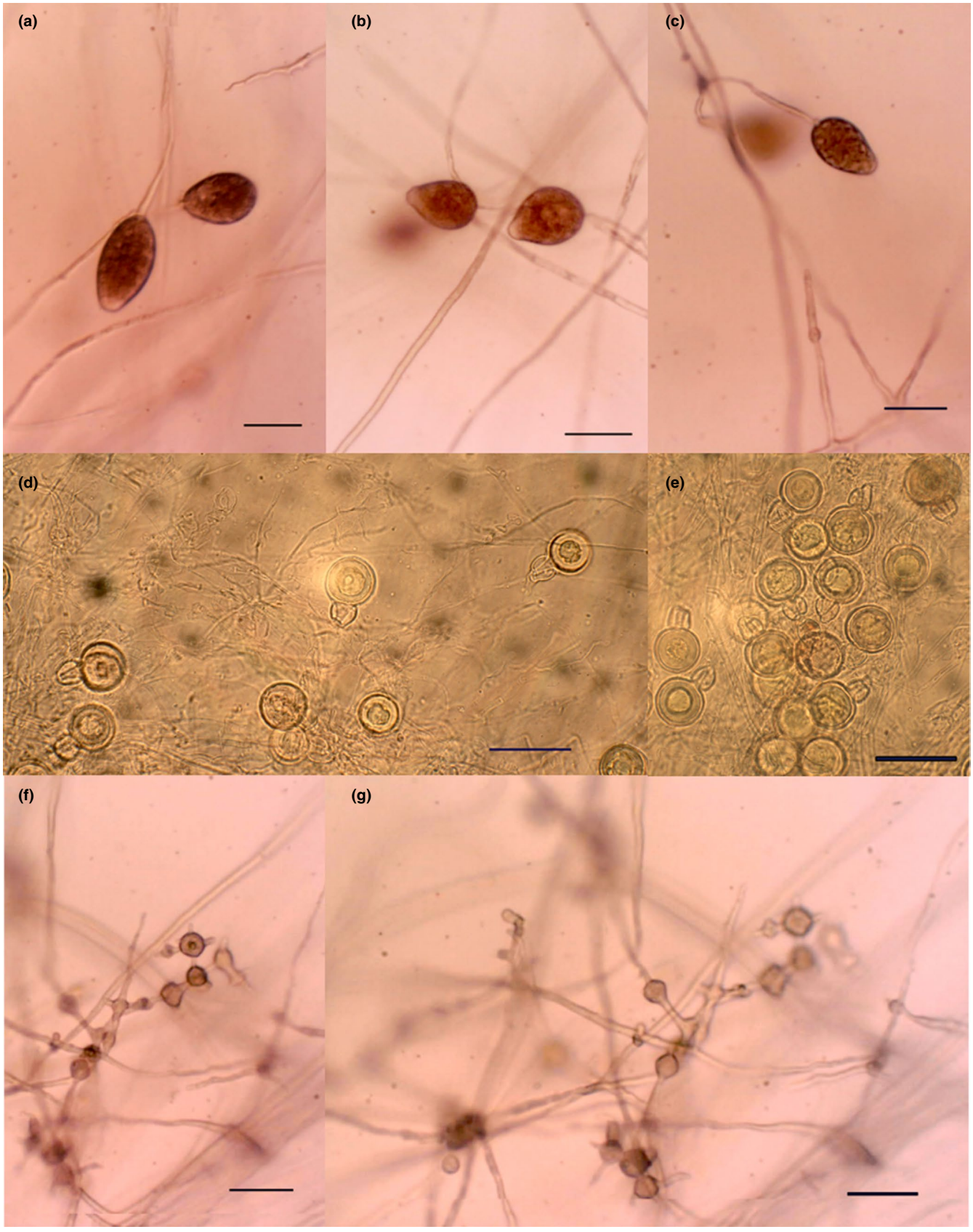

FIGURE 4 *Phytophthora cryptogea*: (a–c) Terminal sporangia, nonpapillate, ovoid to pyriform; (d, e) oogonia and oospores of *P. cryptogea* showing amphigynous antheridial attachment; (f, g) ellipsoid hyphal swellings. Scale bar = 10 μm [Colour figure can be viewed at wileyonlinelibrary.com]

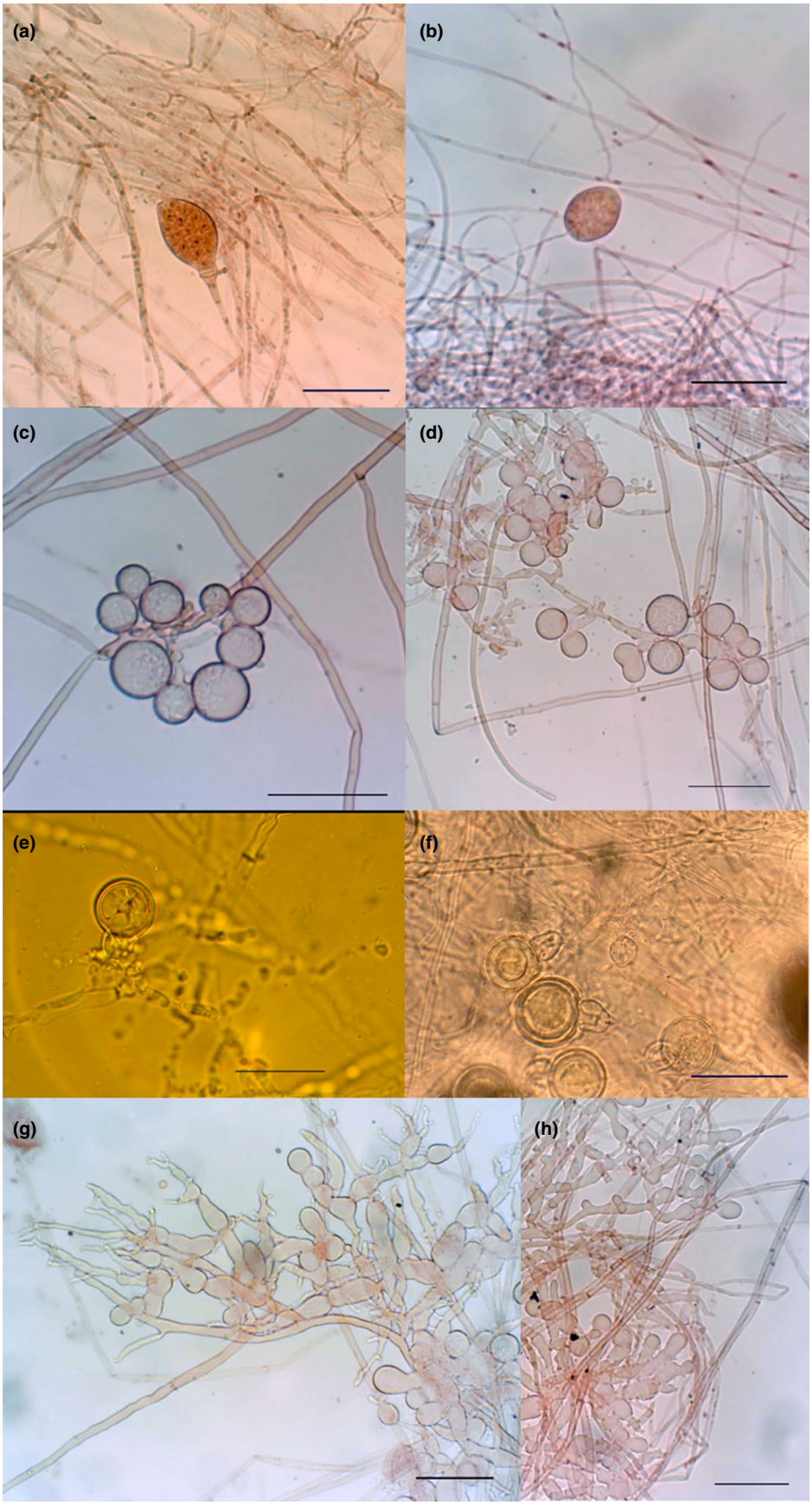

FIGURE 5 *Phytophthora cinnamomi*: (a, b) nonpapillate sporangia; (c, d) globose oogonium with amphigynous antheridium; (e, f) globose chlamydospores, thin-walled and in grape-like clusters of 3–10; (g, h) coralloid hyphae with hyphal swellings. Scale bar = 10 μm [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 4 Analysis of variance of necrotic lesions area carried out for five *Phytophthora* species on detached branches of pomegranate

	Sum of squares	*df*	Mean square	*F*	Sig.
Between groups	29,272,074	5	5,854,414	318.6	<.001
Within groups	440,937	24	18,372		
Total	29,713,011	29			

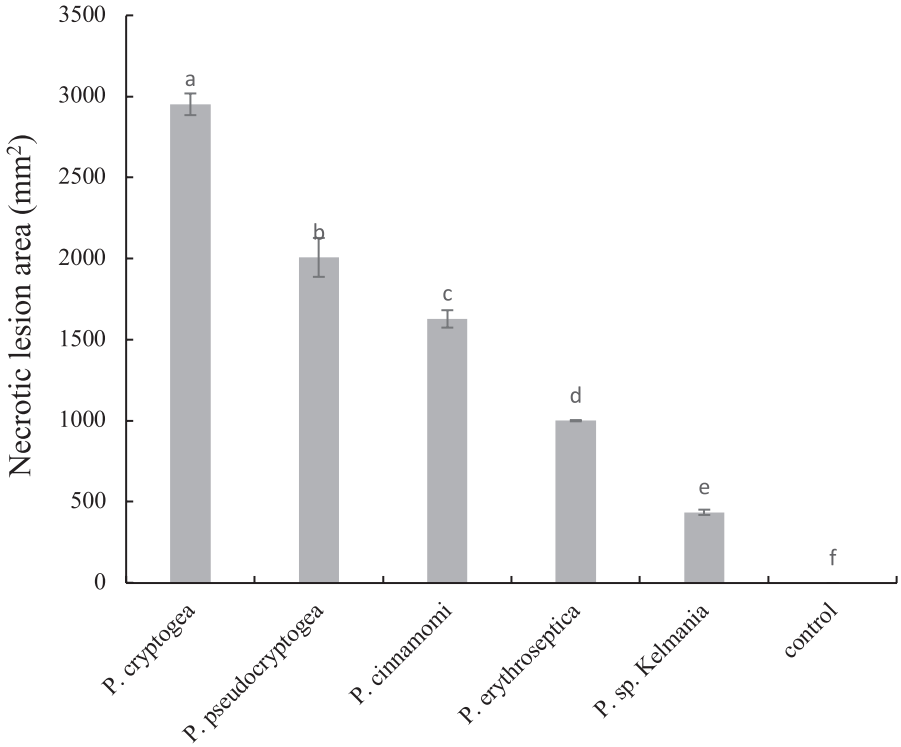

FIGURE 6 Comparison of the mean of necrotic lesions area carried out for five *Phytophthora* species on detached branches of pomegranate using Duncan's multiple range test. Different letters above columns indicate significant differences ($p < .05$)

et al. (2010) suggested *P. erythroseptica* as a secondarily derived homothallic form of *P. cryptogea*, while other phylogenetic analyses of *Phytophthora* species (Martin et al., 2014) considered *P. erythroseptica* as a distinct species. Safaiefarahani et al. (2015) reevaluated the *P. cryptogea* species complex and divided *P. cryptogea* sensu lato into distinct species: *P. cryptogea* sensu stricto, *P. erythroseptica*, and *P.* sp. *kelmania*. Moreover, they described another group of *P. cryptogea* as *P. pseudocryptogea*. In our study, phylogenetic analyses showed that isolates P12 and P13 are *P. cryptogea* sensu stricto. Isolate P14 grouped with *P. pseudocryptogea*, which is morphologically similar to, but phylogenetically distinct from, *P. cryptogea* (Safaiefarahani et al., 2015). Isolate P15 corresponded to *P.* sp. *kelmania* and, consistent with the findings of Martin et al. (2014), formed a separate lineage in the basal position of the Clade 8a phylogenetic tree. Martin et al. (2014) considered *P.* sp. *kelmania* to be clearly separate from *P. cryptogea*. This species has been reported by Abad et al. (2006) but is not yet formally described. Isolates P16 to P23 formed a monophyletic group with *P. erythroseptica* in a distinct clade. Our data is in contrast to Mostowfizadeh-Ghalamfarsa et al. (2010) who suggested that *P. erythroseptica* and *P. cryptogea* are conspecific, but is in agreement with Blair et al. (2008) and Martin et al. (2014) who considered this species distinct from *P. cryptogea*.

The presence of *Phytophthora* species on pomegranate root and crown rots in orchards was expected, because most orchards in the sampled areas have been traditionally irrigated by flooding. This type of irrigation increases the length of saturation, which promotes the discharge and dispersal of zoospores and thereby is conducive to the development of *Phytophthora* root and crown rots (Matheron & Mircetich, 1985).

Our results showed that the time of year was important in isolation of *Phytophthora* species from pomegranate. We were not able to isolate the disease agents from tissues with symptoms and cankers from late winter through to early spring, but they were efficiently recovered between May and September.

The most common species isolated in this study was *P. cinnamomi*, one of the world's most devastating species (Burgess et al., 2017), which was isolated in Isfahan province. This species has a wide host range and has been reported from the root zone of 31 genera of plants in 16 families, including woody shrubs and herbs (Zentmyer, 1983). The existence of this species in Isfahan province poses a threat to other agricultural crops. Climate change issues such

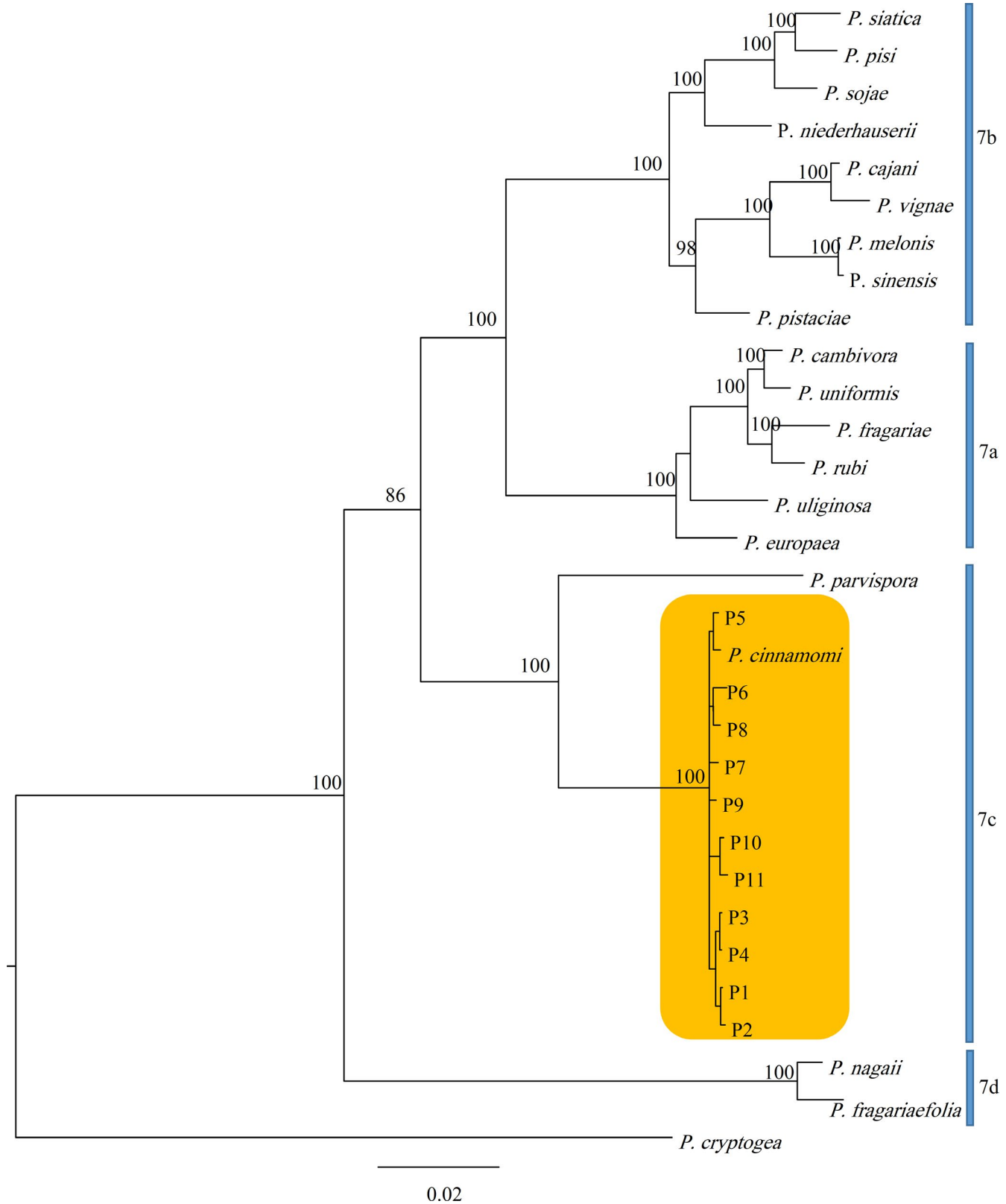

FIGURE 7 Phylogram derived from Bayesian inference analysis of *tub2*, *cox1*, and ITS-rDNA data set of Clade 7 of *Phytophthora*. Bayesian posterior probabilities (in %) are indicated above the branches. *P. cryptogea* is used as outgroup taxon. Scale bars indicate 0.02 changes per site per branch [Colour figure can be viewed at wileyonlinelibrary.com]

as global warming have been reported as an important factor affecting the incidence of *P. cinnamomi* by making hosts more susceptible (Burgess et al., 2017). The introduction of this species into new plant hosts and native vegetation can be a major issue for the country,

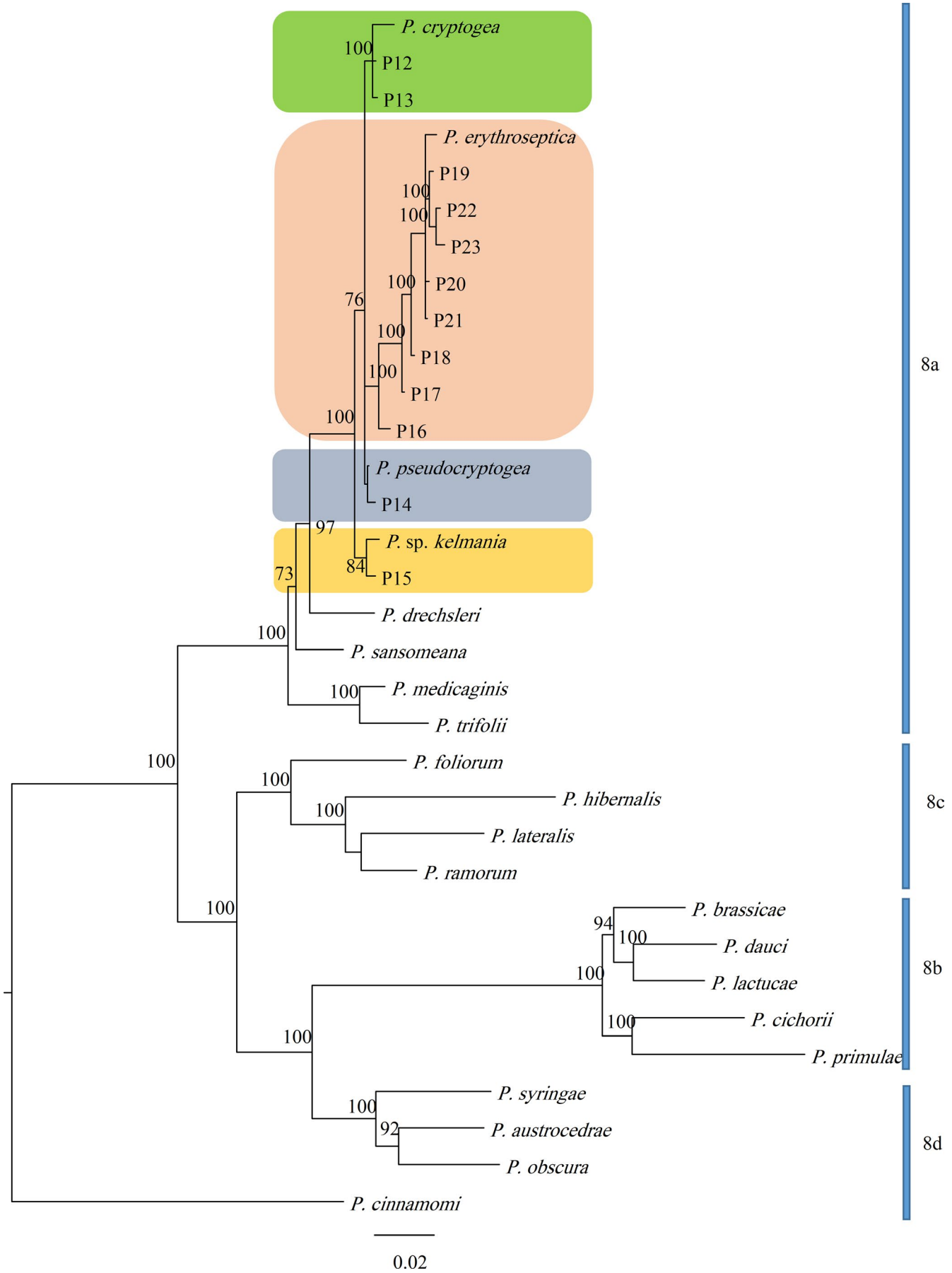

FIGURE 8 Phylogram derived from Bayesian inference analysis of *tub2*, *cox1*, and ITS-rDNA dataset of Clade 8 of *Phytophthora*. Bayesian posterior probabilities (in %) are indicated above the branches. *P. cinnamomi* is used as outgroup taxon. Scale bars indicate 0.02 changes per site per branch [Colour figure can be viewed at wileyonlinelibrary.com]

and management strategies are needed to prevent spreading of the species. This species is reported from *Cedrus* sp., *Chamaecyparis lawsoniana*, *Eucalyptus camaldulensis*, *Persea americana*, and *Quercus ilex* from Iran (Ershad, 2009).

P. cryptogea sensu lato from Clade 8a of *Phytophthora* has a worldwide distribution and causes disease on roots of approximately 50 plant families (Burgess et al., 2009; Delshad et al., 2020). In our investigation, *P. cryptogea* sensu stricto, *P. pseudocryptogea*, *P. erythroseptica*, and *P.* sp. *kelmania*, which are morphologically similar to *P. cryptogea* sensu lato, were isolated in Markazi, Kohgiluyeh va Boyer-Ahmad, and Fars provinces from pomegranate. Our results are consistent with Delshad et al. (2020) who reported that some plant species might be susceptible to both *P. cryptogea* and *P. pseudocryptogea* due to their close phylogenetic relationship. In Kohgiluyeh va Boyer-Ahmad, pomegranate orchards are surrounded by raspberries and *P. erythroseptica* is reported as a causal agent of root rot of raspberry (Converse & Schwartze, 1968). We hypothesized that *P. erythroseptica* may be a mutual pathogen of pomegranates and raspberries in the area, and this needs further investigations on genetic diversity between isolates from the two hosts. *P. cryptogea* sensu lato has been reported from several hosts from Iran (Ershad, 2009). However, the host range and many reports of *P. cryptogea* sensu stricto is uncertain because of the description of species such as *P. pseudocryptogea* and *P. parsiana* that were previously identified as *P. cryptogea* (Mostowfizadeh-Ghalamfarsa et al., 2008; Safaiefarahani et al., 2015).

We isolated *P.* sp. *kelmania* in Fars province, which along with *P. pseudocryptogea* affects pomegranate trees. This is a member of Clade 8a of *Phytophthora* and shares morphological traits with *P. cryptogea* and *P. pseudocryptogea* (Safaiefarahani et al., 2015).

The source of primary introduction of these *Phytophthora* species to pomegranate orchards is not known. Human activities are accepted as a major mode of introduction of fungal plant pathogens (Brasier, 2008). Poor nursery practices such as dense plantings, reuse of planting mixtures or plastic pots without sterilization, and cross-infections resulting from close vicinity of various seedlings species, result in introduction and spread of *Phytophthora* species (Jung et al., 2016). It is possible that these *Phytophthora* species have been in the orchards for a long time. Environmental parameters such as seasonal temperature fluctuations, annual rainfall variations, and global warming are factors affecting the incidence of *Phytophthora* spp. (Burgess et al., 2017; McKeever & Chastagner, 2016). However, intensive sampling of orchards in many regions would help to recognize which other *Phytophthora* species currently reside in the orchards.

Our data showed that *P. cryptogea* was the most virulent species in terms of production of necrotic lesion area on detached branches, followed by *P. pseudocryptogea*, *P. cinnamomi*, *P. erythroseptica*, and *P.* sp. *kelmania*. *P. cryptogea* has high potential to threaten the native Iranian pomegranate orchards. Fortunately, this species has lower occurrence than *P. cinnamomi* and *P. erythroseptica* and is limited to orchards in Markazi province. Therefore, precautionary measures should be attempted to monitor the rootstock transfers between different provinces in order to prevent subsequent spread of these pathogens. The results obtained in laboratory experiments on detached branches cannot explain the extent to which these species are different in causing disease in a host plant in nature, but differences found in pathogenicity can delineate the species differences in terms of aggressiveness. It should be noted that the data presented here for pathogenicity of the species are still preliminary, and further investigation with more isolates in a repeated trial is required.

The information from this study is beneficial to growers by providing information about the pathogens threatening pomegranate trees, as well as other crops that can be new potential hosts for the *Phytophthora* species discussed here. We suggest purchasing *Phytophthora*-free seedlings for planting to avoid seedling-borne diseases, by examining the seedlings for the presence of any *Phytophthora* spp. Correcting the irrigation systems and limiting periods of soil saturation in orchards in order to reduce the dispersal of zoospores to healthy trees is also important. We suggest future surveys look at sources of *Phytophthora* spp. in nurseries in Iran.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest regarding the publication of this paper.

DATA AVAILABILITY STATEMENT

Sequence data were deposited in GenBank and the accession numbers are given in Table 2. Other data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Abad, Z.G., Abad, J.A., Cacciola, S.O., Pane, A., Faedda, R., Moralejo, E. et al. (2014) *Phytophthora niederhauserii* sp. nov., a polyphagous species associated with ornamentals, fruit trees and native plants in 13 countries. *Mycologia*, 106, 431–447.

Abad, Z.A., Abad, J. & Creswell, T. (2006) Species of *Phytophthora* and *Pythium* identified in a long-term collection from North Carolina. *Phytopathology*, 96, S1–S2.

Banihashemi, Z. (1998) Etiology of pomegranate decline in Fars province of Iran. *Phytopathologia Mediterranea*, 37, 127–132.

Bazargani-Gilani, B., Tajik, H. & Aliakbarlu, J. (2014) Physicochemical and antioxidative characteristics of Iranian pomegranate (*Punica granatum* L. cv. Rabbab-e-Neyriz) juice and comparison of its antioxidative activity with *Zataria multiflora* Boiss essential oil. *Veterinary Research Forum*, 5, 313–318.

Blair, J.E., Coffey, M.D., Park, S.Y., Geiser, D.M. & Kang, S. (2008) A multi-locus phylogeny for *Phytophthora* utilizing markers derived from complete genome sequences. *Fungal Genetics and Biology*, 45, 266–277.

Brasier, C.M. (2008) The biosecurity threat to the UK and global environment from international trade in plants. *Plant Pathology*, 57, 792–808.

Burgess, T.I., Scott, J.K., McDougall, K.L., Stukely, M.J., Crane, C., Dunstan, W.A. et al. (2017) Current and projected global distribution of *Phytophthora cinnamomi*, one of the world's worst plant pathogens. *Global Change Biology*, 23, 1661–1674.

Burgess, T.I., Webster, J.L., Ciampini, J.A., White, D., Hardy, G.E.St.J. & Stukely, M.J. (2009) Re-evaluation of *Phytophthora* species isolated during 30 years of vegetation health surveys in Western Australia using molecular techniques. *Plant Disease*, 93, 215–223.

Cacciola, S.O. & Gullino, M.L. (2019) Emerging and re-emerging fungus and oomycete soil-borne plant diseases in Italy. *Phytopathologia Mediterranea*, 58, 451–472.

Çeliker, N.M., Uysal, A., Çetinel, B. & Poyraz, D. (2012) Crown rot on pomegranate caused by *Coniella granati* in Turkey. *Australasian Plant Disease Notes*, 7, 161–162.

Converse, R.H. & Schwartze, C.D. (1968) A root rot of red raspberry caused by *Phytophthora erythroseptica*. *Phytopathology*, 58, 56–59.

Delshad, D., Mostowfizadeh-Ghalamfarsa, R. & Safaiefarahani, B. (2020) Potential host range and the effect of temperature on the pathogenicity of *Phytophthora pseudocryptogea* and its close relatives. *Journal of Plant Pathology*, 102, 753–763.

Ershad, D. (2009) *Fungi of Iran*, 3rd edition. Tehran: Iranian Research Institution of Plant Protection.

Erwin, D.C. & Ribeiro, O.K. (1996) *Phytophthora: Diseases Worldwide*. St Paul, MN, USA: American Phytopathological Society Press.

Hajebrahimi, S. & Banihashemi, Z. (2011) Host range of *Phytophthora parsiana*: a new high temperature pathogen of woody plants. *Phytopathologia Mediterranea*, 50, 159–165.

Huang, Q., Zhu, Y.Y., Chen, H.R., Wang, Y.Y., Lei, Y.L., Lu, W.J. et al. (2003) First report of pomegranate wilt caused by *Ceratocystis fimbriata* in Yumman, China. *Plant Disease*, 87, 1150.

Jeffers, S.N. & Martin, S.B. (1986) Comparison of two media selective for *Phytophthora* and *Pythium* spp. *Plant Disease*, 70, 1038–1043.

Jung, T., Orlikowski, L., Henricot, B., Abad-Campos, P., Aday, A., Aguín Casal, O. et al. (2016) Widespread *Phytophthora* infestations in European nurseries put forest, semi-natural and horticultural ecosystems at high risk of *Phytophthora* diseases. *Forest Pathology*, 46, 134–163.

Khosla, K. & Bhardwaj, S.S. (2013) Occurrence and incidence of important diseases of pomegranate in Himachal Pradesh. *Plant Disease Resources*, 28, 5–10.

Kroon, L.P.N.M., Bakker, F.T., Van den Bosch, G.B.M., Bonants, P.J.M. & Flier, W.G. (2004) Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. *Fungal Genetics and Biology*, 41, 766–782.

Levin, G.M. (1994) Pomegranate (*Punica granatum*) plant genetic resources in Turkmenistan. *Plant Genetic Resources Newsletter*, 97, 31–36.

Markakis, E.A., Tzima, A.K., Palavouzis, S.C., Antoniou, P.P. & Paplomatas, E.J. (2017) First report of *Phytophthora palmivora* causing fruit rot on pomegranate in Greece. *Plant Disease*, 101, 1060.

Martin, F.N., Blair, J.E. & Coffey, M.D. (2014) A combined mitochondrial and nuclear multilocus phylogeny of the genus *Phytophthora*. *Fungal Genetics and Biology*, 66, 19–32.

Matheron, M.E. & Mircetich, S.M. (1985) Influence of flooding duration on development of Phytophthora root and crown rot of *Juglans hindsii* and Paradox walnut rootstocks. *Phytopathology*, 75, 973–976.

McKeever, K.M. & Chastagner, G.A. (2016) A survey of *Phytophthora* spp. associated with *Abies* in U.S. Christmas tree farms. *Plant Disease*, 100, 1161–1169.

Mostowfizadeh-Ghalamfarsa, R., Cooke, D.E.L. & Banihashemi, Z. (2008) *Phytophthora parsiana* sp. nov., a new high-temperature tolerant species. *Mycological Research*, 112, 783–794.

Mostowfizadeh-Ghalamfarsa, R., Panabieres, F., Banihashemi, Z. & Cooke, D.E.L. (2010) Phylogenetic relationship of *Phytophthora cryptogea* Pethybr. and Laff and *P. drechsleri* Tucker. *Fungal Biology*, 114, 325–339.

Murray, M. & Thompson, W.F. (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research*, 8, 4321–4325.

Pethybridge, G.H. & Lafferty, H.A. (1919) A disease of tomato and other plants caused by a new species of *Phytophthora*. *Scientific Proceedings of the Royal Dublin Society*, 15, 487–505.

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D.L., Darling, A., Höhna, S. et al. (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, 61, 539–542.

Safaiefarahani, B., Mostowfizadeh-Ghalamfarsa, R., Hardy, G.E.St.J & Burgess, T. (2015) Re-evaluation of the *Phytophthora cryptogea* species complex and the description of a new species, *Phytophthora pseudocryptogea* sp. nov. *Mycological Progress*, 14, 108–120.

Somasekhara, Y.M. (1999) New records of *Ceratocystis fimbriata* causing wilt of pomegranate in India. *Plant Disease*, 83, 400.

Stamps, D.J., Waterhouse, G.M., Newhook, F.J. & Hall, G.S. (1990) *Revised Tabular Key to the Species of* Phytophthora. CABI Mycology Institute. Mycology Paper no. 162.

Swofford, D.L. (2002) *PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods)*. Sunderland, MA, USA: Sinauer Associates.

Thomidis, T. (2015) Pathogenicity and characterization of *Pilidiella granati* causing pomegranate diseases in Greece. *European Journal of Plant Pathology*, 141, 45–50.

Thomidis, T. & Exadaktylou, E. (2011) First report of *Pilidiella granati* on pomegranate with symptoms of crown rot in the prefecture of Xanthi, Greece. *Plant Disease*, 95, 79.

Turkolmez, Ş., Ciftci, O., Serce, C.U. & Derviş, S. (2016) First report of *Phytophthora palmivora* causing crown and root rot on pomegranate (*Punica granatum* L.) in Turkey. *Plant Disease*, 100, 227.

Tziros, G.T. & Tzavella-Klonari, K. (2008) First report of verticillium wilt of pomegranate caused by *Verticillium dahliae* in Greece. *Journal of Plant Pathology*, 90, 589.

Waterhouse, G.M. (1963) *Key to the species of* Phytophthora *de Bary*. Mycology Paper no. 92. Kew, UK: Commonwealth Mycological Institute.

White, T.J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. (Eds.) *PCR Protocols: A Guide to Methods and Applications*. New York, NY, USA: Academic Press, pp. 315–322.

Yang, X., Tyler, B.M. & Hong, C. (2017) An expanded phylogeny for the genus *Phytophthora*. *IMA Fungus*, 8, 355–384.

Zentmyer, G.A. (1983) The world of *Phytophthora*. In: Erwin, D.C., Bartnicki-Garcia, S. and Tsao, P.H. (Eds.) *Phytophthora, its Biology, Taxonomy, Ecology and Pathology*. St Paul, MN, USA: APS Press, pp. 1–8.

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