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شناسایی، خصوصیات مورفوفیزیولوژیک و تفاوت‌های ژنتیکی گونه قارچی عامل بیماری سرخشکیدگی شاخه درختان پسته استان کرمان بر اساس توالی‌یابی rDNA-ITS

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چکیده

سابقه و هدف: بیماری سرخشکیدگی شاخه یکی از بیماری‌های مهم درختان پسته استان کرمان است که توسط قارچ‌های مختلفی ازجمله *P. variotii* بوجود می‌آید. هدف از انجام این تحقیق شناسایی قارچ عامل این بیماری در استان کرمان بر اساس خصوصیات مرفولوژیکی و توالی‌یابی ناحیه rDNA-ITS بود. امکان وجود تفاوت‌های ژنتیکی در این ناحیه و وجود ارتباطاتی بین تفاوت‌های ژنتیکی با تفاوت‌های مرفولوژیکی و سرعت رشد قارچ در شرایط آزمایشگاهی بررسی شد.

مواد و روش‌ها: در طی سال زراعی ۱۴۰۰، نمونه‌برداری از شاخه‌های بیمار ارقام مختلف پسته باغات استان کرمان انجام گرفت. مراحل جداسازی، کشت، خالص‌سازی و شناسایی گونه قارچ عامل بیماری بر اساس خصوصیات مرفولوژیک انجام شد. تکثیر و توالی‌یابی ناحیه ITS با استفاده از آغازگرهای ITS1 و ITS4 انجام شد. سرعت رشد جدایه‌های قارچ روی محیط کشت ثبت و میانگین رشد آن‌ها محاسبه و مقایسه گردید. با به‌کارگیری نرم‌افزار مگا۱۰، درخت فیلوژنتیکی ترسیم شد.

یافته‌ها: بر اساس خصوصیات مورفولوژیکی و توالی‌یابی ITS، تمامی جدایه‌ها، گونه *P. variotii* شناسایی شدند. درخت فیلوژنتیکی، وجود تفاوت ژنتیکی در ناحیه ITS جدایه‌ها را نشان داد. ارتباط معنی‌داری بین گروه‌بندی فیلوژنتیکی جدایه‌ها با تفاوت‌های مورفولوژیک و میانگین سرعت رشد مشاهده نشد.

نتیجه‌گیری: تفاوت‌هایی در توالی‌های ناحیه ITS در سطح درون گونه‌ای وجود دارد. توالی‌یابی ITS، پشتیبان ارزشمندی برای تایید مشاهدات مورفولوژیکی در جهت شناسایی *P. voraitii* است. ارتباط مستقیمی بین گروه‌بندی فیلوژنتیکی با تفاوت‌های مورفولوژیکی و فیزیولوژیکی جدایه‌ها وجود نداشت.

واژگان کلیدی: بیماری سرخشکیدگی، واکنش زنجیره‌ای پلی‌مراز، ITS1، ITS4، درخت فیلوژنتیک.

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Identification, morpho-physiological characteristics and genetic differences of the fungal species causing pistachio die-back disease in Kerman province based on rDNA-ITS sequencing

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Abstract

Background & Objectives: Dieback disease is a major problem in pistachio trees in Kerman province. It is caused by various fungi, including *P. variotii*. This study aimed to identify this fungal species based on morphology and rDNA-ITS sequencing. The study also investigated genetic differences in this region and correlations between genetic variations, morphology, and fungal growth rate in lab conditions.
Materials & Methods: During 2021, diseased branches from different pistachio cultivars in Kerman were sampled. Isolation, cultivation and purification of the isolates were carried out and identification of the fungal species were performed based on morphological characteristics. The ITS region was amplified and sequenced using specific primers ITS1 and ITS4. The growth rate of fungal isolates on culture media was measured and compared. A phylogenetic tree was constructed using MEGA10 software.
Results: According to morphological characteristics and the ITS sequences of the isolates, all isolates were identified as *P. variotii*. The phylogenetic tree demonstrated genetic differences in the ITS region among the isolates. No significant correlation was observed between the phylogenetic grouping of the isolates and the morphological differences or the average growth rate.
Conclusion: There were differences in the sequences of the ITS region among the isolates. Sequencing the ITS region provided valuable support to confirm the morphological observations to identify *P. variotii*, although it could not establish a direct correlation between phylogenetic grouping and the morphological and physiological differences among the isolates.
Keywords: Dieback disease, ITS1, ITS4, PCR, phylogenetic tree.
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Introduction

Pistachio (*Pistacia vera*) is a member of the Anacardiaceae family and is one of the most important horticultural products in Iran, with a significant contribution to the country's economy. Iran's pistachio production stood at 135,000 tons in 2021, making it the second-largest producer after the United States, which produced 523,000 tons in the

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same year. More than 125,544 hectares of pistachio orchards are harvested in Iran, with approximately 73% located in Kerman Province. Over 50% of Iran's pistachio production is exported, making it a major export for the country (1).

Dieback disease is one of the most significant diseases affecting pistachio orchards in Kerman province in Iran. The first observation of dieback disease in pistachio trees was reported in 1987 by Aminaee and Ershad in Kerman Province (2). This disease has spread widely in pistachio orchards and caused a sharp reduction in production in recent years (3,4). Aminaee and Ershad (1987) reported the disease agent, *Paecilomyces variotii*, by morphological investigations and demonstrating pathogenesis of the fungi isolated from infected branches of pistachio trees (2).

Alizadeh et al. (2000) reported that the contamination of dieback disease in the Rafsanjan region of Iran was in the range of 0-85%. Until now, more than 53 fungal species have been reported on pistachio trees from different countries (5). Among these, nine species can create the canker, burning and drying symptoms in twigs. These species include: *Botrytis cinerea* (6), *Sclerotinia sclerotiorum* (7), *Pellicularia koleroga*, *Phomopsis* sp. (8), *Rhizoctonia solani*, *Cladosporium* sp., *Alternaria* sp., *Fusarium* sp. and *Phoma* spp. (9). Dieback disease in Iran has been reported to be caused by several pathogenic agents, including: *P. variotii* (3,4,10,11), *Seimatosporium fusisporum* (12), *Cytospora*sp. (3,4), *Bacillus licheniformis* (13), *Coniotyrium* sp., *Alternaria* sp., *Ulocladium* sp., *Fusarium equiseti* and *Nattrassia mangiferae* (4). In a study by Sohrabi et al. in 2020, *Botryosphaeria dothidea*, *Diplodia seriata*, *Eutypella citricola*, *Phaeoacremonium minimum*, *Phaeoacremonium parasiticum* and *Phaeoacremonium viticola* fungal species were identified as the cause of branch and trunk dieback disease in pistachio trees across different regions of Iran (14).

In addition to fungi, some bacterial species have also been reported as agents of pistachio branch dieback and canker diseases. In Chile and Iran, the bacterium *Pantoea agglomerans* is reported as the cause of the pistachio dieback and canker diseases, respectively (15,16).

In various countries, different fungal species have been identified as the cause of this disease. Recently, *Fusarium proliferatum* has been identified as the main causal agent of pistachio dieback and canker disease in Turkey (17). In another study, it was reported that *Neoscytalidium novaehollandiae* associated with pistachio dieback in the Southeastern Anatolia region of Turkey (18). *Botryosphaeria dothidea, Lasiodiplodia pseudotheobromae, Neofusicoccum mediterraneum, N. parvum, Diaporthe neotheicola, Diaporthe sp., Eutypa lata, Eutypa sp., Cytospora sp., and Phaeoacremonium minimum* have been identified as branch dieback, panicle and shoot blight of pistachio in southern Spain (19). Research conducted in Italy has shown that *Botryosphaeria dothidea*, *Neofusicoccum hellenicum*, and *N. mediterraneum* fungal species causing diseases such as shoot dieback, cankers, fruit spots, and leaf lesions in pistachio trees (20).

Many of these studies, have used morphological characteristics to identify the fungal species. In Iran, researchers have consistently relied on morphological characteristics for the identification of Paecilomyces species. Consequently, *P. variotii* has been identified as the exclusive causal agent of dieback disease on *Pistacia vera*.

In the field of biotechnology sciences, the sequencing of different regions of DNA has become a widely applicable method for classifying organisms and identifying species (21). It has proven to be a valuable tool for investigating the evolutionary relationships among organisms (22).

Ribosomal DNA (rDNA) genes are commonly used markers for determining molecular phylogenetic relationships. The rDNA gene cluster consists of three exon regions (18S, 5.8S, and 28S) and two intron regions (ITS and IGS) (23). This cluster is the main locus to study phylogenetic relationships. The sequences of rDNA genes have fixed frequencies of conserved and varied sequences within the genome. IGS and ITS regions of rDNA evolve faster than the other regions and the sequences of the ITS region are stable and show only slight differences within a species. These characteristics make rDNA genes valuable for studying phylogenetic relationships. The presence of multiple copies of rDNA sequences per genome and the pattern of concerted evolution among repeated copies facilitate their analysis.

The ITS region is a helpful tool for studying phylogenetic relationships among neighbor species, within species, and/or among close species. (24). Also, The ITS region is an appropriate region to confirm morphological observations used for identification of species. In their study, Luangsa-ard and Hywel-Jones (25) utilized 18S rDNA sequence data to conduct a phylogenetic analysis of the genus Paecilomyces. Their findings revealed that the genus is polyphyletic. Additionally, they highlighted the morphological variation observed in the type species, *P. variotii*. The *P. variotii* species complex has been identified as consisting of five distinct species: *P. divaricatus*, *P. formosus*, *P. brunneolus*, *P. dactylethromorphus*, and *Byssochlamys spectabilis* (considered the sexual state of *P. variotii*) (26, 27). Among these, *P. formosus* exhibits possible differentiation into three separate species, namely *P. formosus*, *P. lecythidis*, and *P. maximus*. However, distinguishing these three taxa solely based on microscopic characteristics and extrolite analyses is not possible, and molecular phylogeny data is required.

Although the ITS region of the genome is known to exhibit conserved sequences within a species, minimal differences have been observed among different isolates of the same species. In relation to certain fungal species, a correlation has been reported between morphological and physiological variations along with differences in the sequences of this genomic region. In *Alternaria alternata*, the causal agent of tangerine brown spot, genetic differences in ITS sequences among different isolates were reported. Additionally, a positive relationship among differences in growth rates of isolates in vitro and the groupings of isolates based on sequencing of the ITS region was observed (28).

The objective of this study was to identify the causative agent responsible for dieback disease in pistachio branches in Kerman Province using morphological and molecular traits, and exploring genetic variations among different isolates of the pathogen through sequencing of the rDNA-ITS region as well. Furthermore, we investigated potential correlations between morphological and physiological differences, such as growth rate in laboratory conditions, among different isolates of the fungal pathogen and the observed variations in the ITS region sequences. Notably, this research represents the first investigation into such relationships regarding the fungal pathogen associated with dieback disease.

Materials and Methods

A: Sampling and isolation of fungus: During 2021, samples were collected from pistachio orchards of different areas of Kerman Province. The collected samples consisted of twigs from different varieties of pistachio trees that showed symptoms of drying and were suspected to be diseased (Table 1). In total, 18 samples were collected. The samples were placed inside plastic bags and immediately transferred to the plant pathology laboratory of Islamic Azad University branch of Jahrom. The infected twigs were washed with tap water to remove any external contaminants or debris.

Table 1: Isolate names, sampled regions, variety names and number of collected isolates.

Isolate names	location	Variety name	No. of isolates
Pv1	Zarand	Akbari	3
Pv2	Zarand	Ahmad Aghai	3
Pv3	Sirjan	Fandoghi	3
Pv4	Kerman	Kaleghochi	3
Pv5	Rafsanjan	Momtaz	3
Pv6	Ravar	Momtaz	3
Total			18

Then, parts of the junction between healthy and infected tissues of twigs were removed using a sterile surgical blade and surface disinfected using 2% sodium hypochlorite solution for 2 min. The sterilized pieces were placed on potato dextrose agar (PDA) medium in Petri dishes and incubated at 25°C. After 48–72 h, the obtained fungal colonies were purified using the single-spore method.

B: Growth rate of isolates: The purified isolates were inoculated on PDA medium, and each isolate was placed in separate Petri dishes. These dishes were then transferred to an incubator set at a temperature of 25°C. The experiment followed a completely randomized design with three replications for each isolate. The fungal colony diameter was measured and recorded from the underside of the Petri dishes every 48 h for one week. The average linear growth rate of isolates (ALGR) was calculated using the following formula:

$$ALGR\ (mm/d) = (C_n - C_1)/\ (n - 1)$$

where Cn is colony diameter on the last day, C1 is colony diameter after 1d of culture and n is the number of days (29).

C: Identification of species based on morphological characteristics: First, the purified fungal colonies were cultured on Potato Carrot Agar (PCA) and PDA media to investigate the morphological characteristics and identify species. After 5d of culturing at 25°C, the fungal colony, colour was recorded from the upper and lower sides of PDA medium. Characteristics of conidiophores, conidia, phialides and chlamydospores were investigated and recorded on PCA medium.

The key for identifying species of Paecilomyces was used to determine the species of the isolates (30). The key identified Paecilomyces species by evaluating characteristics like colony colour, conidiophore morphology, conidial shape and size, and chlamydospore presence in the isolates.

D: DNA extraction: Young fungus mycelia (cultivated on PDA medium for 24 h) were used for DNA extraction. Initially, the mycelia of each isolate were carefully collected from the culture media using a sterile surgical blade and transferred to a sterile pestle. The mycelia were frozen using liquid nitrogen prior to grinding them in the sterile pestle. Subsequently, the CTAB method (31) was utilized for various extraction steps. To assess the concentration and purity of the obtained DNA, a NanoDrop device (Biowave II) was

employed (Biochrom Company United Kingdom).

E: Amplification of ITS region: The ITS1 / ITS4 primer sets were employed for amplification of the complete ITS region (22). The PCR amplification conditions consisted of initial denaturation for 5 min at 94 °C, followed by thirty-one cycles of 35 s at 94 °C, 55 s at 54 °C, and 1 min at 72 °C, plus a final extension of 15 min at 72 °C. The PCR reaction utilized the Accupower PCR premix master mix (Bioneer Company, South Korea). Following electrophoresis on a 1.5% agarose gel stained with ethidium bromide, PCR products were visualized under a Gel Doc device after 45 min at 75 V (22).

Table 2: The isolate names and the accession numbers in Gene Bank.

Isolate names	Gene Bank accession no.
Pv1	KP337408.1
Pv2	KP337410.1
Pv3	KP337411.1
Pv4	KP337412.1
Pv5	KP337413.1
Pv6	KP337414.1

Following the appropriate amplification of the ITS region, PCR products were directly submitted for sequencing to Bioneer Company. The acquired sequences were then submitted to the Gene Bank (accession numbers of isolates registered: KP337408–KP337414) and compared with the available sequences (Table 2).

F: Data analysis: SAS software was used for statistical analysis. Average statistical groupings were compared using Duncan's multiple-range test at $P < 0.05$. BLASTn searches were used to compare the obtained sequences from the ITS region of isolates with the existing sequences of Gene Bank. MEGA10 (Molecular Evolutionary Genetics Analysis, Biodesign Institute, USA) software was used to construct the phylogenetic tree of isolates based on the ITS sequences. After alignment, maximum likelihood method was used for phylogenetic reconstruction. Maximum likelihood analysis was performed with the heuristic search option. To assess branch support values, bootstrapping was performed, using 1000 replications. *Paecilomyces formosus* (*Pf*) FJ389921.1, was chosen as the outgroup. Bankit internet software was used to record sequences in the Gene Bank database and assign accession numbers.

Results

A: Morphological identification: The colonies exhibited a consistent color across all samples, ranging from brick-red to brownish, except for the *Pv2* isolate which displayed an olive-green hue. The conidiophores displayed chains of conidia with varying lengths, characterized by smooth walls and oval to spindle shapes. In most samples, the conidial length fell within the range of 2–4 µm, while the width ranged from 4–6 µm. However, the *Pv2* isolate stood out with a conidial length spanning from 4–7 µm, surpassing the measurements of the other samples.

The phialides, observed as integrated or single entities, possessed cylindrical or oval bases that gradually extended into a slender "neck" structure. Chlamydospores were formed either in the middle or at the termination of the mycelia. Upon comparing the morphological characteristics of the isolates with the provided key for identifying Paecilomyces species (27), it was determined that all isolates belonged to the *P. variotii* species.

B: Growth rate of isolates under experimental conditions: The average growth rates among the isolates exhibited significant variations, with *Pv4* and *Pv6* isolates demonstrating the highest and lowest rates of growth, respectively (Figure 1).

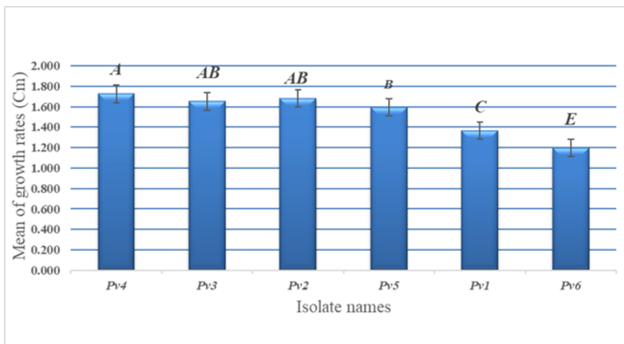

Fig. 1. Mean of growth rates for various *P. variotii* isolates. Dissimilar letters above the columns indicate significant variations according to Duncan's test ($P < 0.05$).

C: Molecular identification of isolates: The amplification of the ITS region in the isolates was successful, resulting in a 580 bp band for all isolates without any observed polymorphism (Figure 2).

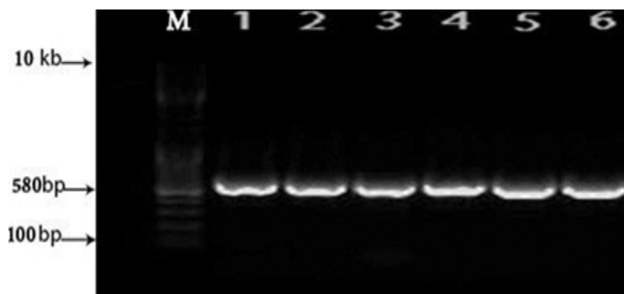

Fig 2. Amplification of the ITS region of *P. variotii* isolates on 1.5% gel agarose. M, marker of Fermentas company 1kb; and lanes 1–6 represent *Pv1-Pv6* isolates, respectively.

Comparison of the obtained ITS sequences with those in the Gene Bank revealed that the isolates exhibited the highest similarity to the species *P. variotii*, *P. formosus*, and *P. sinensis* (Table 3).

Table 3: The similarity percentages of sequences for the ITS region of the isolates with the sequences in Gene Bank.

Similarity percentage			Name of isolate
P. sinensis	*P. variotii*	*P. formosus*	
98	98	98	*Pv1*
99	99	99	*Pv2*
99	99	99	*Pv3*
99	99	99	*Pv4*
99	99	99	*Pv5*
98	98	99	*Pv6*

The similarity percentage between the investigated isolates and the existing ITS sequences of *P. variotii*, *P. formosus*, and *P. sinensis* in the Gene Bank ranged from 98% to 99% (Table 3). Considering the significant similarities and the morphological characteristics of the isolates, they were conclusively identified as *P. variotii*.

D: Phylogenetic tree based on ITS sequences: The phylogenetic tree constructed from the sequences of ITS region divided the investigated isolates into two main groups (clades) (Figure 3). The first group included the *Pv4* and *Pv5* isolate and were generally distinct from other isolates. The second group consisted of secondary sub-groups with *Pv1* and *Pv3* isolates and *Pv2* and *Pv6* isolates were placed in another sub-group (Figure 3). The results showed genetic differences in ITS region sequences at the intra-species level among the isolates from different varieties of pistachio.

Discussion

The morphological and molecular studies conducted in this research unequivocally demonstrated that all isolates examined were identified as *P. variotii*. Previous morphological investigations carried out by various

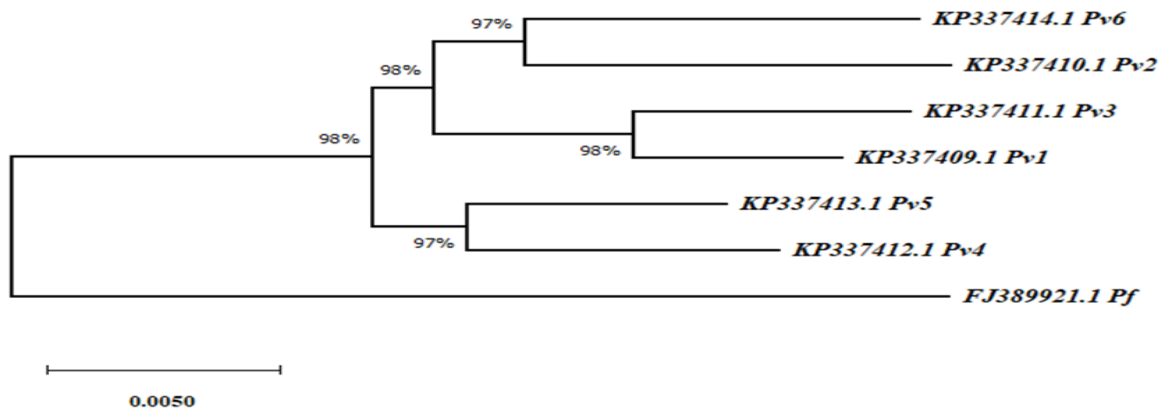

Fig 3. Phylogenetic tree based on ITS-rDNA region sequences of six isolates of *P. variotii* using the Maximum-likelihood method. The numbers above each branch indicate the amount of bootstrap support from 1000-time phylogenetic tree drawing using the Maximum-likelihood method. *Paecilomyces formosus* (*Pf*) (FJ389921) species is an outgroup sample.

researchers also supported the attribution of *P. variotii* as the causal agent responsible for dieback disease in pistachio trees within Kerman Province (2,10). Furthermore, sequencing analysis of the ITS region of these isolates provided additional confirmation, aligning with prior studies in Iran that employed morphological approaches to identify the fungal species (2,10).

The findings of this research highlight the prominent role of *P. variotii* as the primary causative agent of pistachio twig dieback disease in Kerman Province. In their study, Alizadeh et al. (2000) thoroughly investigated the aetiology of this disease in pistachio trees within Iran and identified *Paecilomyces variotii* as the responsible pathogen (4). Additionally, Ghelichi et al. (2012), substantiated the pathogenic nature of various Paecilomyces isolates on pistachio through both in vitro and in situ experiments, confirming them as *Paecilomyces variotii* (32). Two distinct species of the genus Paecilomyces have been reported in various host plants across Iran. Specifically, *P. variotii* has been observed in almond, pistachio, and sesame (33), while *P. tenuis* has been identified in wheat (34).

The evolutionary relationships of *P. variotii* isolates were thoroughly examined by conducting sequencing analysis of the ITS region. The findings unveiled significant genetic disparities among *P. variotii* isolates obtained from diverse pistachio varieties. Through the construction of a phylogenetic tree, the analyzed isolates were segregated into two distinct groups, clearly illustrating their genetic dissimilarities. This divergence may be attributed to various genetic events, such as mutations occurring within the sequences of the ITS region specific to this particular isolate. Coincidentally, Peay et al. (2008) reported the occurrence of mutations within this genomic region. Previous studies have similarly highlighted inter-specific variations in the ITS regions and portions of the β-tubulin gene for the identification of Paecilomyces strains in clinical samples (35).

Our findings align closely with the research

conducted by Sabbagh and Khosravi Moghaddam (2016). They collected isolates from pistachio orchards in Iran's Kerman province and morphologically identified them as *Paecilomyces variotii* using ITS-RFLP pattern analysis (36). Similarly, Fargues et al. (2002) reported a significant level of polymorphism within *P. fumosoroseus* isolates sourced from different geographical locations and host insects, based on rDNA-ITS region sequencing. Thus, our results are consistent with both studies (37). However, in this study, all isolates initially identified morphologically as *P. variotii* were subjected to rDNA-ITS sequencing, which revealed that the isolate sequences closely resembled those of *P. formosus*, *P. variotii*, and *P. sinensis*. These findings were anticipated since *P. variotii* is a complex species that can be further classified into five distinct species: *P. divaricatus*, *P. formosus*, *P. brunneolus*, *P. dactylethromorphus*, and *Byssochlamys spectabilis* (the sexual state of *P. variotii*) (26,27).

A comprehensive examination of Byssochlamys and *Paecilomyces variotii*-like isolates by Houbraken et al. (2010) demonstrated that the genus Byssochlamys, along with its related anamorphic species could be categorized into at least nine distinct taxa (27). In a separate study conducted by Heidarian et al. (2018), all isolates collected from pistachio trees exhibiting dieback symptoms were initially identified as *P. variotii*; however, further physiological investigations revealed that some of these isolates actually belonged to *P. formosus*. Moreover, phylogenetic analysis based on DNA variation (ITS, β-tubulin, and calmodulin) indicated that all of these isolates formed a clade together with *P. Formosus* (38).

From these findings, it can be concluded that relying solely on morphology for identifying the causal agent of pistachio dieback is challenging. Therefore, molecular-based identification methods, such as sequencing, offer a more reliable and robust alternative for discriminating between fungal species.

The growth rates of the investigated samples varied significantly under the experimental conditions. Additionally, notable morphological differences were observed in both the size of spores and the colour of colonies among the isolates that were examined. These variations could potentially be attributed to differences in the host variety, as well as the climate and geographical conditions of the sampling location. In fact, certain researchers (26) have reported variations in the growth rate between isolates belonging to the '*P. maximus*-clade' and other members within this diverse group. Furthermore, distinct variations in terms of shape, colour, and reverse characteristics of colonies were also observed among *P. formosus* isolates obtained from diseased pistachio trees in Iran (38).

In the phylogenetic tree, isolates *Pv6* and *Pv4* were assigned to distinct groups, with *Pv6* demonstrating the highest growth rate and *Pv4* exhibiting the lowest growth rate under laboratory conditions. Interestingly, despite their disparate growth rates, isolates *Pv4* and *Pv5* were grouped together in the phylogenetic tree. Conversely, isolates *Pv2* and *Pv3*, which shared similar growth rates, were placed in separate subgroups within the phylogenetic tree. Notably, isolate *Pv2* exhibited a distinct colony colour compared to the other isolates but did not form a separate subgroup in the phylogenetic tree. This suggests that there is no clear correlation between the morphological characteristics of the isolates and their ITS

region sequences. However, a previous study reported a strong association between the morphological characteristics and sequencing of the ITS region in *A. alternata* isolates, the causal agent of tangerine brown-spot disease in Iran. Additionally, a study on *P. formosus* isolates from diseased pistachio trees found a high level of agreement (98%) between physiological traits and phylogenetic trees based on ITS and β-tubulin sequence data. Overall, these findings suggest that while a distinct relationship between the morphological characteristics and ITS sequencing may not exist for *P. variotii* isolates, other studies have demonstrated correlations between these factors in different fungal species. The sequencing results unequivocally validate the efficacy of using the ITS region for precise identification of Paecilomyces species and corroborating the accuracy of morphological observations in species identification but it cannot be utilized to address issues related to morphological and growth rate variations among various Paecilomyces isolates. Further investigation is necessary. Sequencing of other genomic regions, such as IGS and EndoPG, is recommended to establish robust relationships between morphological differences and molecular methods for isolates of this species.

Conclusion

Different species of fungi and bacteria can cause the disease known as pistachio tree dieback. This study specifically found that the fungus *Paecilomyces variotii* is the cause of pistachio tree dieback in Kerman province. In many studies, fungal species are identified based on morphological characteristics. The use of molecular techniques such as ITS sequencing can provide more accurate and precise identification of fungal species, especially in cases where morphological characteristics may overlap among species However, in the present study, in addition to morphological characteristics, the sequencing of ITS region was used for identification of fungal species causing the disease. The results of confirmatory sequencing validated the morphological identification of this fungus, and in both cases, the fungus *Paecilomyces variotii* was identified. The phylogenetic tree constructed based on ITS sequencing showed that there are genetic differences in the sequences of this region among different isolates of this fungus. Also, the results of this study showed significant differences in the morphology and growth rate of different isolates. However, no direct correlation was observed between these morpho-physiological differences and the phylogenetic tree. The study suggests using sequencing of additional genomic regions such as IGS, EF, etc. To gain a deeper understanding of the morpho-physiological relationships among various isolates of this fungus. This approach will help analyze their genetic variations and combine the phylogenetic trees generated from sequencing of these genomic regions.

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Ethical Considerations

The authors of this article have observed all ethical principles, such as avoiding plagiarism, maintaining literary standards, simultaneous publication, and refraining from data manipulation and fabrication.

Conflict of interest

The authors declare that they have no conflicts of interest.

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